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Neuronal Cell Cultures as Toxicologic Test Systems

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Neuronal cell cultures now represent well-characterized systems with which acute and chronic toxicologic effects of a variety of agents can be evaluated. Extensive synapse formation occurs over a period of days and weeks in these cell cultures and can be assayed semiquantitatively by morphological and electrophysiological means. Detailed morphophysiologic correlations can be made using a technique for injecting an intracellular marker protein, horseradish peroxidase. A variety of neurochemical indices of development, such as transmitter-related enzyme levels, can also be conveniently determined. The developing neuron and its synaptic connections are important objects of investigation since they may be particularly vulnerable to pathogenic materials. Examples of the effects of acute (opiate) and chronic (inhibitory amino acid) treatments on synaptic function are given.

One approach to the evaluation of the impact on the nervous system of various toxicologic agents would be to apply these agents directly to central neurons in culture and to assess their effect on a variety of important neurobiologic parameters. In this review I describe a tissue culture preparation from the mammalian central nervous system and to give some indications of the quantitative measurements of neuronal function that can be used to evaluate effects of the substances being evaluated.

Information transfer in the nervous system takes place at the specialized contacts between neurons called synapses. We have been interested in cellular and molecular aspects of synapse formation between neurons of the central nervous system. For studies in this area it was important to have preparations that are as accessible and experimentally manipulable as possible. Obviously, however, one of the requirements of the preparations is that they exhibit synaptic function, and moreover that they develop synaptic function during a period in which we have some degree of control over their environment, since we are interested both in mechanisms of synaptic function and the process of development of those synapses.

The preparation we have employed is the mouse spinal cord in cell cultures (*1, 2*). We begin with fetal material in which the neurons are immature and synapse formation is extremely primitive or nonexistent. Histologic sections of such material, a 13-day-old mouse spinal cord, are shown in Figure 1*a* at relatively low power. The dorsal root ganglion, the dorsal horn and the ventral horn are shown. The cells are relatively undifferentiated, (Fig. 1*b*) and at this stage very little synapse formation has taken place, in contrast to the mature mouse spinal cord where the white matter and gray matter are clearly demarcated, and large neuronal cells can be clearly differentiated from the neuropil (Fig. 1*c-e*). The relatively primitive fetal structure is taken out, minced very finely and dissociated by a variety of procedures in order to obtain a single cell suspension. When this cell suspension is plated onto plastic tissue culture dishes, the cells do not look much like neurons at all; but over a period of two or three weeks they develop into larger cells with very long processes and a clearly neuronal morphology. After about a month, some large spinal cord (SC) cells with multipolar tapering processes, and dorsal root ganglion (DRG) cells, with one or a few processes can be discriminated growing on a background mat of connective tissue and presumed glial cells (Figs. 2 and 3).

Fixation, embedding, thin sectioning and electron microscopy of the cultured cells is readily accomplished, and qualitative differences between spinal cord and dorsal root ganglion cells are evident. The surface membranes of both cell types are

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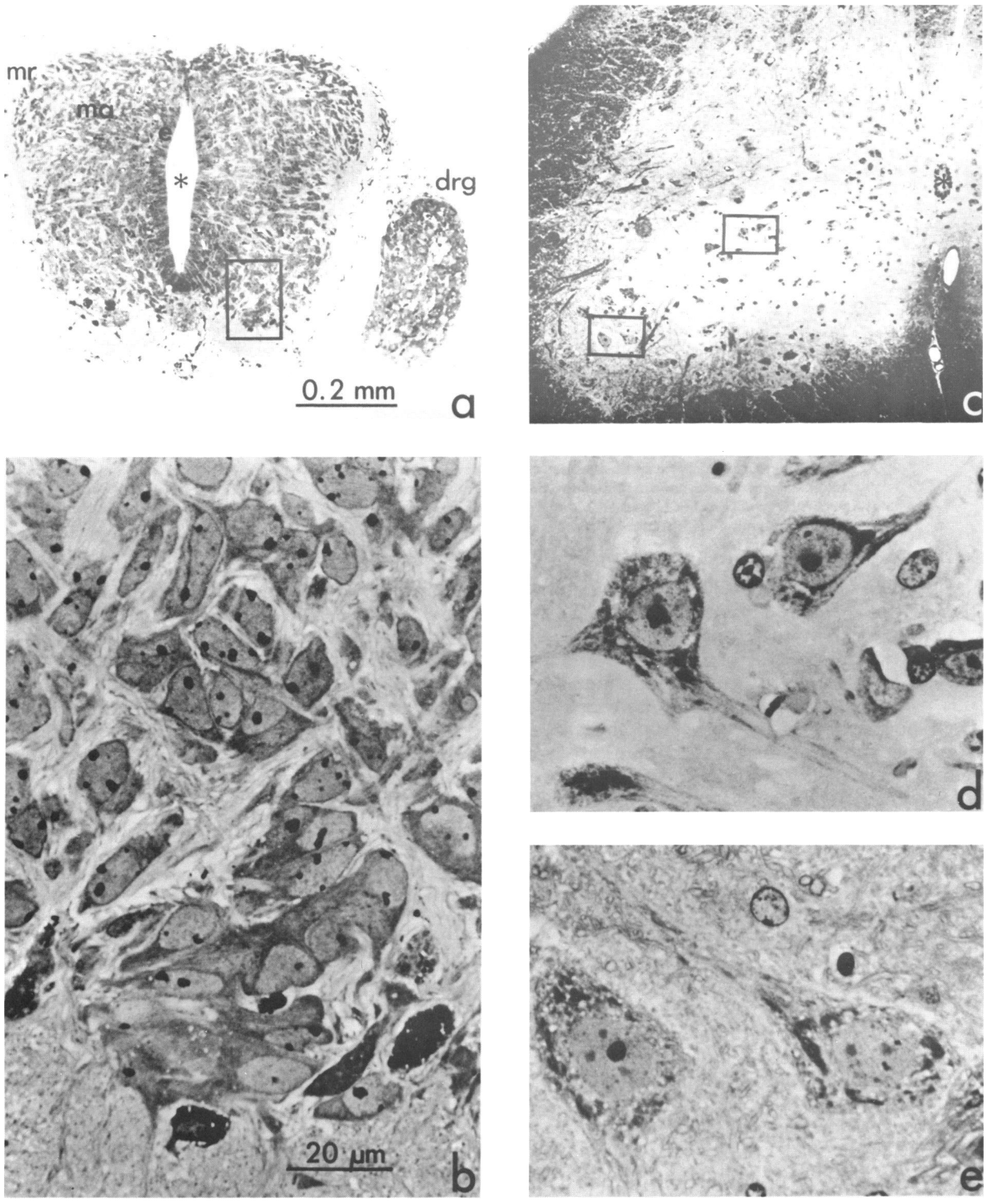

FIGURE 1. Toluidine blue-stained Epon sections through a 12-day fetal (*a, b*) and adult (*c, d, e*) mouse spinal cord: (a) section through entire cord and one dorsal root ganglion (drg), ×100; (*b*) area of basal lamina enclosed by box in (*a*), ×1050; (c) Portion of adult cord, ×100; (*d, e*) anterior horn cells enclosed by boxes in (c), ×1050. Asterisks in (*a*) and (*c*) indicate the central lumen and central canal, respectively. The ependymal lining (e) of the lumen persists in the adult; the embryonic mantle (ma) and marginal (mr) layers become the adult gray and white matter. From Ransom et al. (*2*).

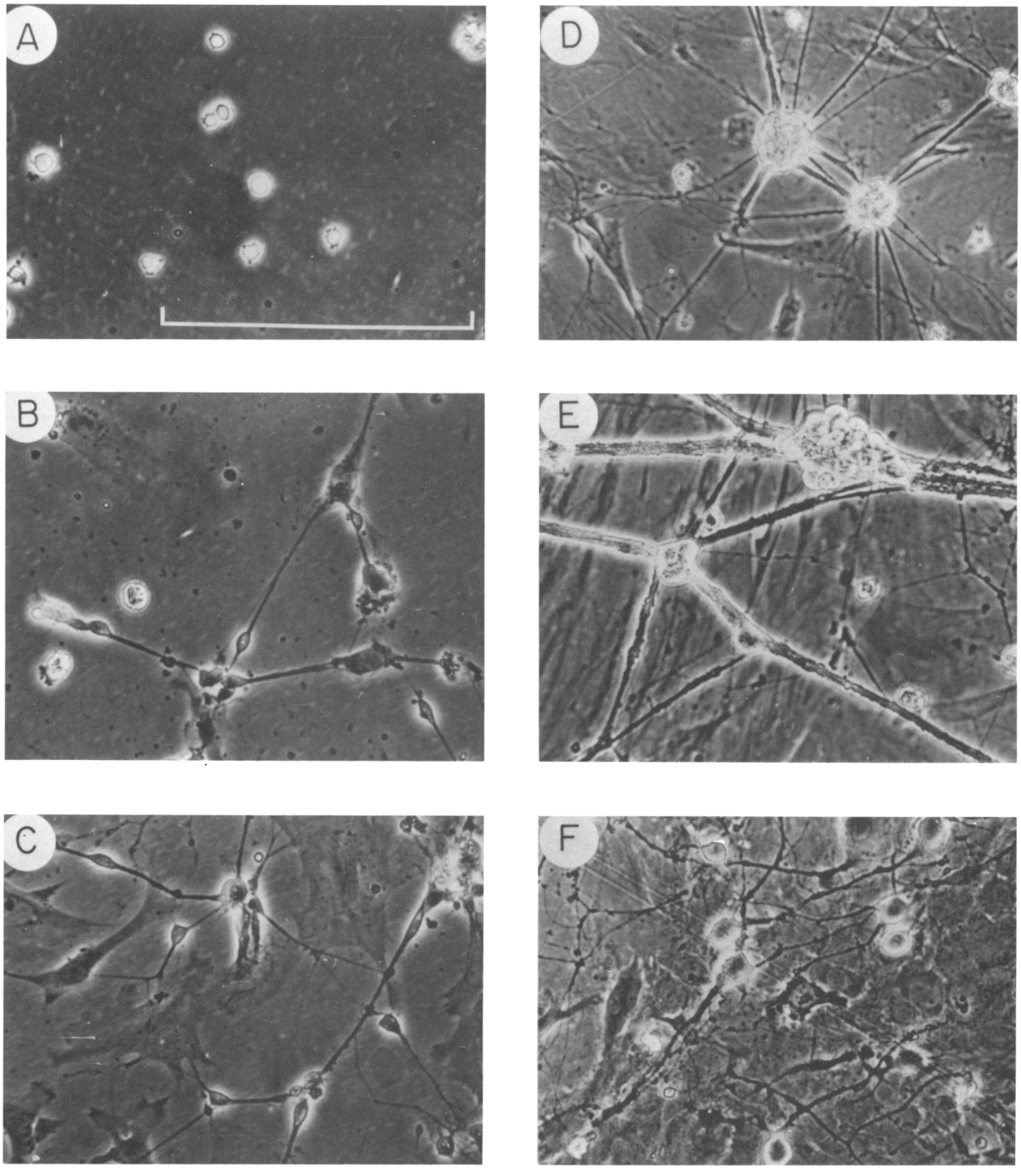

FIGURE 2. Composite of phase contrast photomicrographs of various culture stages: (*A*) dissociated cord and ganglion cells from 11-day-old fetus, cells were attached to collagen-covered surface of dish about 6 hr after plating; similar microscopic fields were seen at each fetal age used; (B) cells with long processes 24 hr after plating, surface was collagen-covered, fetal gestational age 14 days; (*C*) same culture as *B*, 72 hr after plating and after 48 hr of incubation in medium containing aminopterin; note flat background cells; (*D*) higher magnification from 1-wk-old culture growing on collagen-covered surface, fetal gestational age 11 days; (*E, F*) same dish as *C* after 4 wk in culture. Calibration bar is 200 μm for *A, B, C, E,* and *F*; for D, it is 275 μm. From Peacock et al. (*1*).

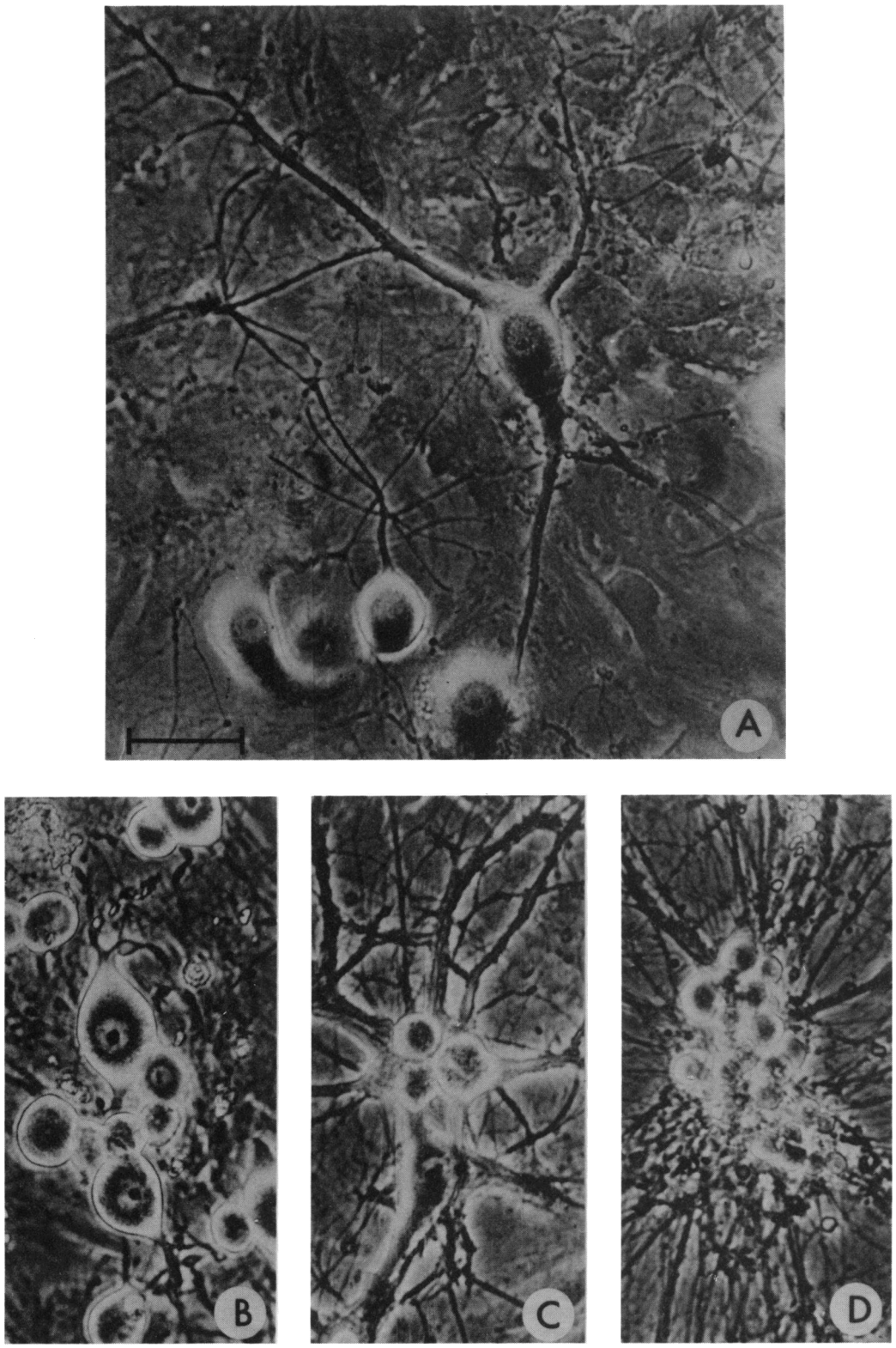

FIGURE 3. Phase photomicrographs of living mouse spinal neurons in cell culture, illustrating the variety of cell aggregates which are most commonly studied in mature cultures: (A) Isolated large SC (right) and DRG (lower left) cells; (*B*) group of DRG cells; (*C*) small aggregate of SC cells; (*D*) larger aggregate of SC cells which form a clump. The marker in *A* indicates 50 μm and applies to all panels. From Ransom et al. (*2*).

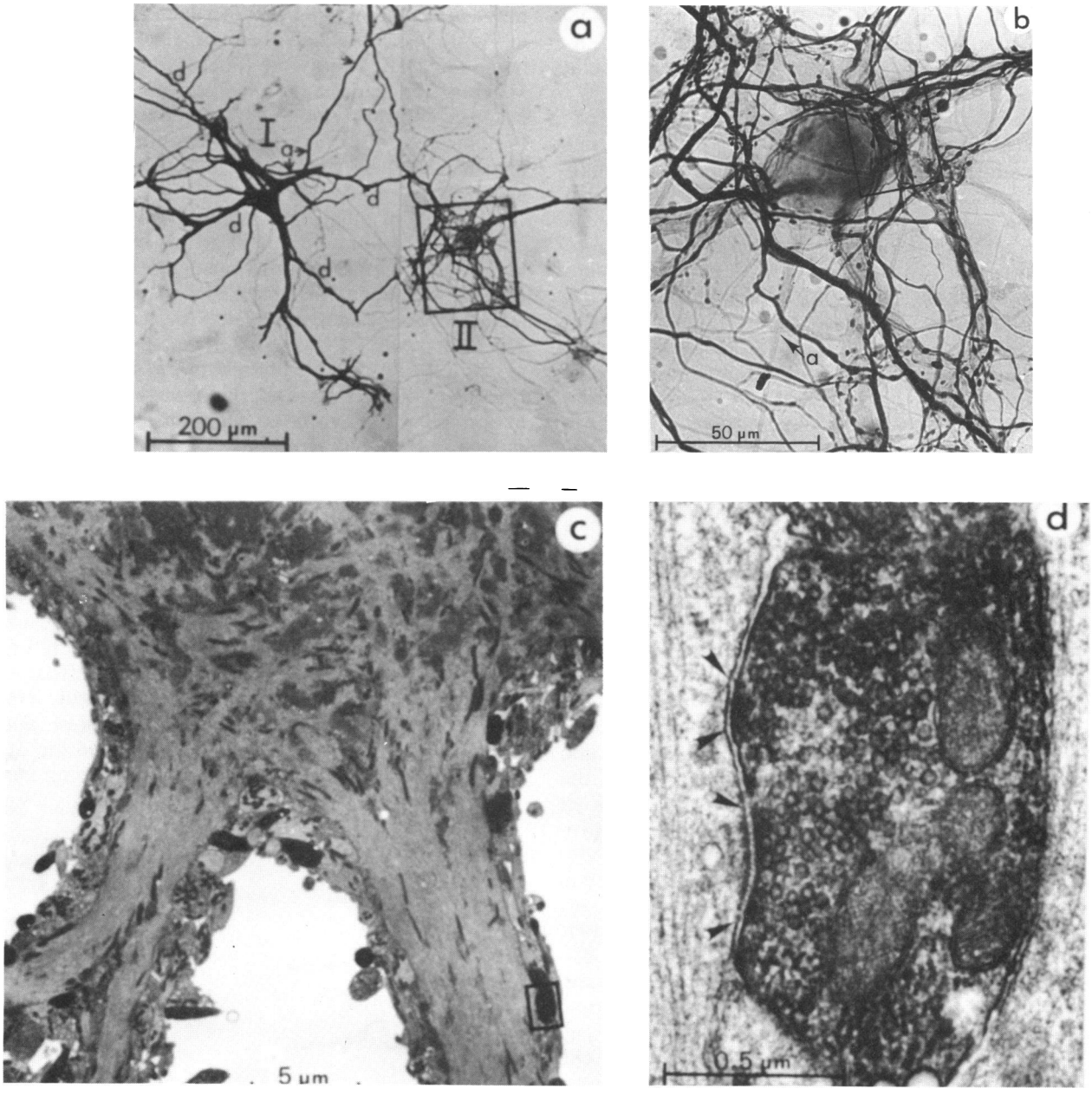

FIGURE 4. Light (*a*, *b*) and electron (*c*, *d*) photomicrographs of a cultured spinal cord neuron injected with horseradish peroxidase (HRP). (a) Intracellular transmembrane potential recordings showed that current injection into cell I produced an action potential which elicited a 20 mV excitatory postsynaptic potential (EPSP) in cell II. Cell I was subsequently injected by intracellular iontophoresis with 4% HRP, and 10 hr later, fixed and reacted for the enzymes. Several rather smooth-surfaced, tapering dendrites (d) emerge from the pyramidal soma of the injected cell. A single axon (a) arises from the base of a large dendrite and branches once (arrows) almost immediately. One of these axonal branches further divides and enwraps the soma and dendrites of cell II. Several additional large neurons (not shown) are similarly contacted by processes of cell I; one such cell receiving a dense morphologic investment is 1.5 mm distant from cell I. ×120. (b) Boxed area of cell II; HRP-labeled axonal branches entwine the soma and dendrites of cell II. Numerous labelled swellings, many of which appear to contact cell II, are visible in this field: (a) axon of cell II. ×800. (*c*) The boxed area of (*b*), rotated clockwise by ~90°. The surface of cell II is contacted by many processes and boutons, although those derived from cell I, containing HRP, are easily discerned because of their increased electron density. ×4000. (*d*) The boxed terminal in (*c*). This HRP-labeled terminal contacts a large dendrite of cell II. Such terminals contain round vesicles and multiple synaptic complexes (limited by arrowheads). Postsynaptic dense material, when seen, is not pronounced. × 62,000. Kindly supplied by Dr. Elaine Neale.

in immediate contact with the culture medium although the spinal cord cells are substantially encrusted with synaptic contacts while the DRG cells are free of such contacts.

In order to study these neurons electrophysiologically, the tissue culture dishes are put on the stage of an inverted microscope so that multiple microelectrodes can be introduced under direct vision to record intracellularly from the cells and to apply neuroactive materials to the cells iontophoretically. The neurons are as expected, electrically excitable, and there are somewhat subtle, but nonetheless diagnostic electrophysiologic differences between spinal cord (SC) and dorsal root ganglion (DRG) cells in addition to the morphologic differences (*3*, *4*). The dorsal root ganglion cell's action potential has a calcium component, and both calcium and sodium ions flow across the membrane during the action potential. One can partially block the DRG cell spike by low sodium or tetrodotoxin containing solutions, but some spike activity persists in such solutions, and in the presence of an increased calcium ion concentration fairly normal looking action potentials occur. Action potentials in SC cells, however, are completely blocked by tetrodotoxin and are due essentially completely to a sodium mechanism.

The cell cultures are characterized by a high degree of synaptic interactions between the constituent neurons (*5*, *6*). Combined anatomical and electrophysiological methods allow analysis of the synaptic connections in some detail. Pairs of neurons can be impaled with intracellular microelectrodes, and, in a substantial proportion of contiguous pairs of neurons, action potentials elicited in one neuron produce synaptic potentials in the other. Impaled neurons can be injected with a marker, horseradish peroxidase (HRP), which serves to display the full anatomical extent of the nerve cell and to allow discrimination at the electron microscopic level of all the synaptic boutons of the injected cell (*7*) (Fig. 4). Thus correlation between the physiological and pharmacological character of a given synaptic connection (excitatory, glycinergic inhibitory, GABAergic inhibitory) and light and electron microscopic structural features is feasible. In order to obtain a more quantitative assessment of synaptic activity in these cultures one can use a statistical approach. The nature of synaptic transmission has been much explicated by studies at the neuromuscular junction; in this and essentially every other place that has been examined carefully the basic nature of chemically mediated synaptic transmission is quantal. That is, there are least units of synaptic activity that can be identified which are probably related to the discharge of a single synaptic vesicle, and evoked activity such as that shown in Figure 5 is composed of a number of these quanta being released at the same time as a result of a presynaptic action potential. We wanted to be able to describe central synaptic action in these terms, and to quantify it in terms of mean quantal size (q) and the mean quantal number m involved in any postsynaptic potential.

This sort of quantitation has involved computer analysis of the amplitudes of a long series (several hundred) of serially elicited postsynaptic potentials (PSPs). From the variance and mean amplitude of such a series of synaptic events the q and m values can be determined. Typically, the size of the unitary synaptic event is some 200 μV (*6*). When we correlate the number of quanta released by a presynaptic action potential with the number of boutons involved in the synaptic contact, it appears that each bouton releases, on the average, about one quantum or vesicle for each presynaptic action potential (*7*).

An experiment dealing with the mechanism of action of opiates illustrate the utility of this quantal analysis of central synaptic action (*8*). We asked if the potent opiate, etorphine, would influence the function of the synapse between dorsal root ganglion cells and spinal cord cells in our culture system and, if so, whether the opiate effect is primarily on the presynaptic or postsynaptic element. DRG-SC cell pairs were impaled and amplitude histograms of the evoked PSP in the SC cell obtained under control conditions, during ion-

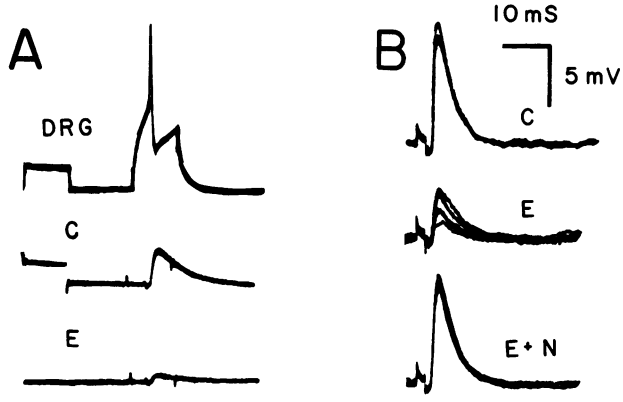

FIGURE 5. Etorphine antagonizes the DRG to SC EPSP. DRG stimulation (5 msec duration depolarizing current) evoked a typical action potential (*A-DRG*) followed by a 10-12 mV EPSP recorded in the SC with a 2.5 msec latency (*A-C* and *B-C*). Iontophoresis of etorphine HCl (1*mM*, 5 nA) produced clear attenuation in EPSP amplitude (*A-E* and *B-E*) and iontophoresis of naloxone HCl during etorphine application at a point adjacent to the etorphine pipet reversed this antagonism, restoring the EPSP to 80% of its control value (*B-E+N*). Cal pulse in *A* is 10 mV × 5 msec. From Macdonald and Nelson (*8*).

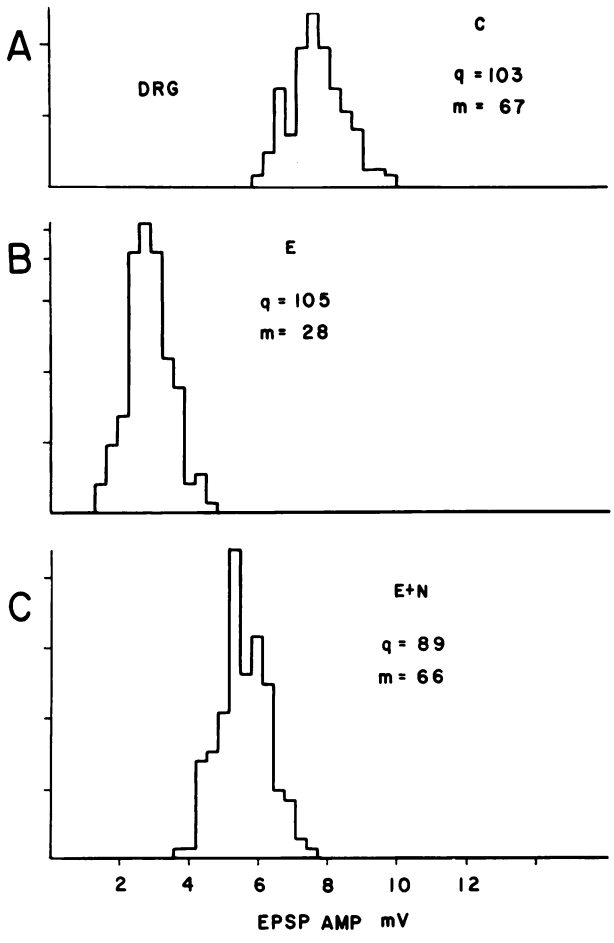

FIGURE 6. Etorphine produces naloxone reversible presynaptic inhibition of DRG transmitter release. Evoked EPSP amplitude histograms: (*A*) during a control run, (*B*) during steady application of etorphine HCl (5 nA), and (*C*) during coincident etorphine and naloxone (30 nA) iontophoresis. The values for quantal size q in microvolts and quantal content m were calculated by using the variance method (7) and were corrected for nonlinear summation of the EPSPs and for system noise (assuming an rms noise level of 300 μV). The results demonstrate presynaptic inhibition of transmitter release (decrease in m) with minimal postsynaptic effect (no change in q), an effect which was largely naloxone reversible. The ordinate scale represents 10 responses per division. From Macdonald and Nelson (*8*).

tophoretic application of etorphine, and under the combined effect of etorphine and the specific opiate antagonist, naloxone. Quantal size and quantal number were derived from these data as shown in Figures 5 and 6. The data showed that the opiate had essentially no effect on quantal size but substantially reduced the average number of quanta involved in each PSP. The opiate effect was reversed by naloxone. The size of the quantal synaptic event is determined largely by postsynaptic factors, or at least a change in postsynaptic sensitivity would be reflected in a change in quantal size. The quantal number on the other hand is determined primarily by the presynaptic release mechanism. The results therefore argue for a presynaptic locus of action for opiates as far as the DRG-SC synaptic linkage is concerned.

A number of such acute neuropharmacological studies are feasible with the cell culture system, but in some ways the cultures lend themselves most naturally to more chronic long term studies. We typically maintain the cultures for 6–8 weeks before using them for physiological or anatomical studies and they survive quite well up to 4 months. Direct exposure of the neuronal surface to any agent for long periods is easy to arrange. What are the assays of neural function that can be used as indices of the action of any test material?

A study we have done on inhibitory synapse formation illustrates some of these points (*9*). We

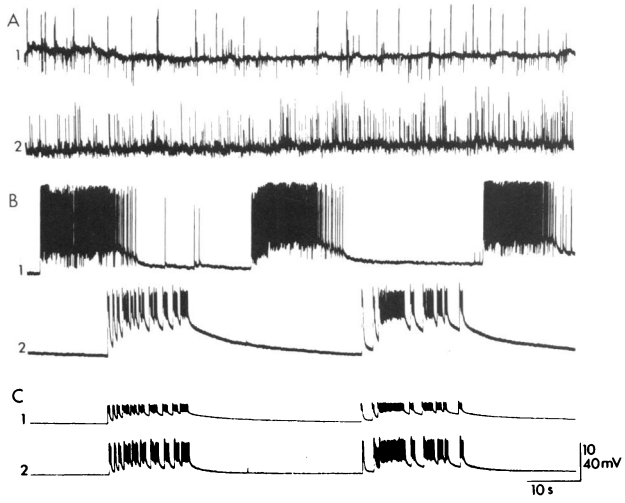

FIGURE 7. Patterns of spontaneous activity in SC cells grown and tested in different media. (A1, A2). Two different SC cells grown and tested in MEM (cell in 2 was originally grown in MEM plus glycine medium, but was switched to MEM alone 1 wk prior to this recording). Activity patterns are typical of cells grown under these conditions and consist of high-frequency synaptic input, IPSPs, and EPSPs, with occasional excitatory potentials leading to spike discharge. (B1, B2). Two different SC cells exhibiting burst-pattern spontaneous activity. This is characterized by powerful, synchronized cascades of excitatory synaptic input, usually in the absence of IPSPs. Note the relatively quiet base line between bursts. Recording B1 is from cell grown in MEM plus 0.4*mM* glycine and switched to MEM alone just prior to recording. Recording B2 is from cell grown and recorded in DMEM (which contains 0.4*mM* glycine). (C1, C2) Simultaneous recordings from two different SC cells grown and tested in DMEM. Note the precise synchrony of the bust discharges seen in these cells, which was a reliable feature of adjacent neurons in neural populations showing well-developed bust discharge. Recording B2 is a high-gain record of C1. All resting potentials were greater than 50 mV. Calibration = 40 mV for *A* and *B*; 10 mV for *C*. From Nelson et al. (*9*).

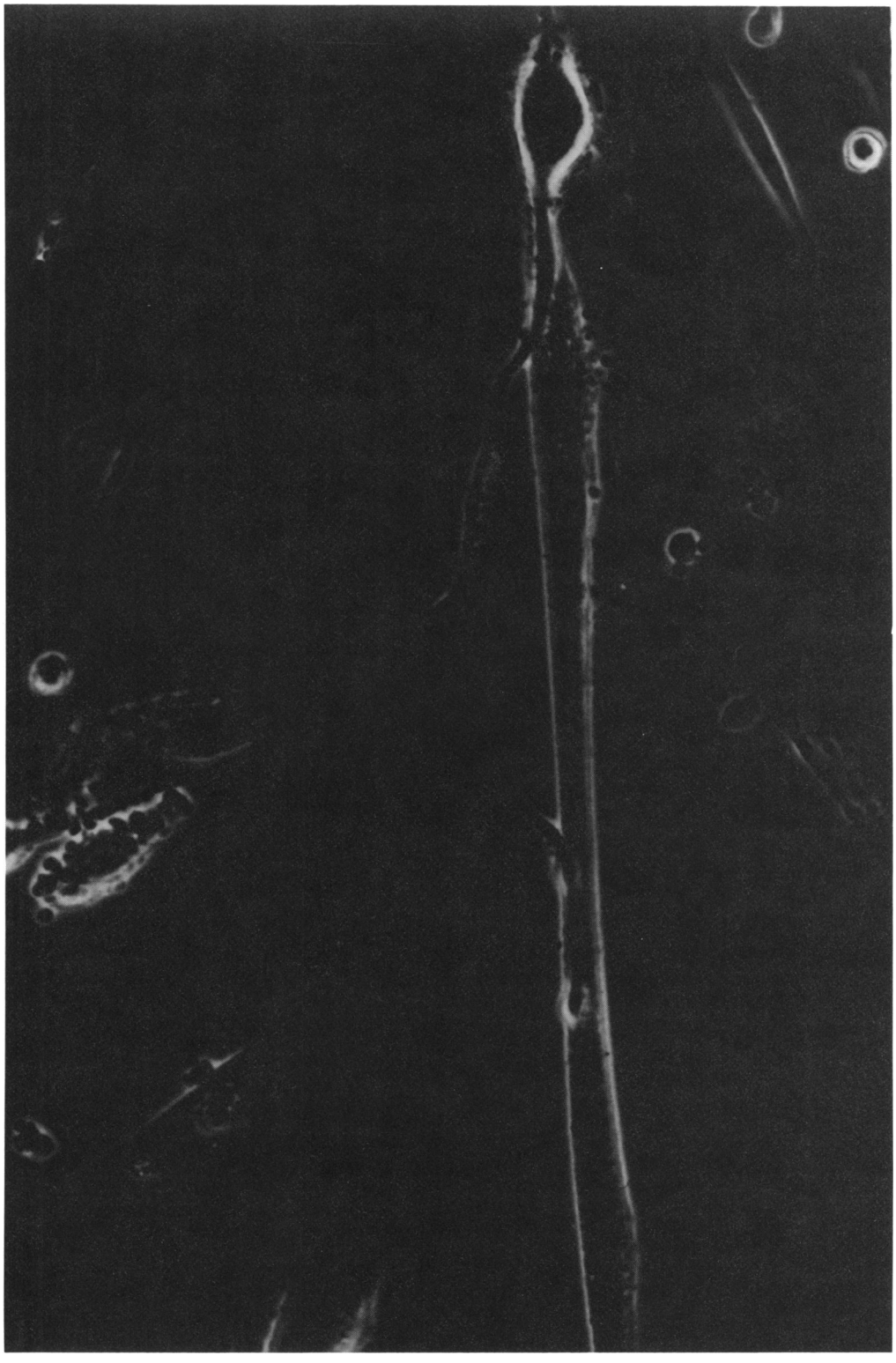

FIGURE 8. Clonal neurons and myogenic cells. The neuronal cells are a hybrid line, the NG108-15, formed between neuroblastoma and glioma lines and the muscle cells are a continuous line derived from the mouse. The figure shows a co-culture plated 31 days previously with myotubes and 18 days previously with hybrid cells. From Christian et al. (*12*).

wanted to determine the effect of the long-term action of inhibitory amino acids on neural function and development and to this end raised sister control cultures, cultures grown in glycine, and cultures grown in γ-aminobutyric acid (GABA). Our assay was electrophysiologic and consisted of recording from many cells and counting the incidence of cells in which excitatory post-synaptic potentials (EPSPs), inhibitory post-synaptic potentials (IPSPs), or bursts of spike and synaptic activity could be documented. The changes in the pattern of activity produced by chronic glycine treatment are shown in Figure 7. Rather than the more or less continuous asynchronous activity characteristic of normal culture activity, bursts of paroxysmal depolarizing synaptic activity with high frequency action potentials were commonly seen in cultures raised in the presence of glycine. These bursts were separated by periods of nearly total quiescence. Cultures raised in the presence of GABA, on the other hand, were generally very quiet. In only a very small percentage of the sampled cells raised in glycine did we see any evidence of inhibitory synaptic activity. We think it likely that this paucity of synaptic inhibition was responsible for the bursty, paroxysmal character of the ongoing neuroelectric activity seen in those cultures. Both the deficit in inhibitory synaptic and the paroxysmal spontaneous activity persisted for at least 6 hr after the glycine-raised cultures were changed to normal medium. Within 48 hr of such a change, however, the physiologic picture was normal.

More detailed studies of synaptic physiology could be done in such long term experiments as well as anatomical or biochemical studies (such as transmitter related enzyme levels) (*10, 11*). A profile of neurobiologic development can be generated to capture best the features of relevance to the problem under consideration.

This presentation has focussed on essentially one preparation with which we have had a good deal of experience. A number of other neuronal cell culture systems are available, each of which has distinctive properties and advantages. An example of a simpler synaptic system is shown in Figure 8. This consists of a presynaptic neuron and a postsynaptic muscle cell each of which belongs to a cloned line of cells (*12*). The cells in these clones divide continually when passaged and large amounts of material can be generated. A variety of methods are available to produce cessation of mitosis and the expression of a number of differentiated neurobiologic properties, including synapse formation (*12, 13*). In addition, the field of explant cultures is one with a great deal of promise for toxicologic work (*14*). The literature dealing with this variety of preparations should be consulted for further details (*15, 16*).

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